

Rapid presumptive diagnosis of gonococcal urethritis in men by the limulus lysate test

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SUMMARY In an evaluation of the limulus assay as a method for detecting endotoxin in urethral exudates, positive results for urethral samples at a 1/200 dilution were obtained from 73 out of 73 patients with culture-positive gonococcal urethritis while negative results were obtained from 26 out of 27 patients with culture-negative urethral specimens. A specimen from one patient, which gave negative results on Gram stain and culture, gave positive results to the limulus test. The overall accuracy of the limulus test for predicting culture results was 99% ($P < 0.001$). Thus, in preliminary studies of otherwise healthy men, the results of the limulus assay correlated with those of biological methods for diagnosing urethral gonorrhoea; the test may, therefore, be of use in identifying cases of nongonococcal urethritis.

Introduction

Studies in recent years (Melton, 1976; Felman and Scaffidi, 1977) have shown that gonorrhoea is the most common, reportable infectious disease in the United States. In 1976, there were one million reported cases of gonorrhoea (Venereal Disease Control Division, 1976), over 600 000 occurring in men. Rapid and sensitive diagnostic methods are needed in the management of patients with gonococcal and nongonococcal urethritis (NGU). A variety of techniques for diagnosing gonococcal disease have been developed (Welch and O'Reilly, 1973; Jacobs and Kraus, 1975; Feng *et al.*, 1977) but most are complex and generally require unavailable materials.

Pioneer studies (Levin and Bang, 1964) showed that a lysate from washed amoebocytes of the *limulus polyphemus*, the horseshoe crab, formed a gel in the presence of minute amounts of endotoxin elaborated by Gram-negative bacteria. The limulus assay has been used for detecting endotoxin in blood (Levin *et al.*, 1970), urine (Jorgensen *et al.*, 1973), and joint and spinal fluids (Tuazon *et al.*, 1977), and as a method for pyrogen testing of injectable commercial fluids (Jorgensen and Smith, 1973). The use of the limulus endotoxin assay in the diagnosis of

gonococcal urethritis and as a possible means of identifying cases of NGU is the subject of this report.

Material and methods

STUDY POPULATION

One hundred men with uncomplicated urethritis seen at the Columbus Health Department Venereal Disease Clinic were studied. These patients had sought treatment because of urethral discharge or dysuria or both and were selected on a random basis; only those with a purulent urethral discharge or a discharge obtained after urethral massage were included. Patients receiving antibiotic therapy within 10 days of presentation were excluded. The mean age was 23.6 years and the range, 19-28 years. The patients presented one to nine days after noticing symptoms, with a mean of 2.8 days.

DIAGNOSTIC PROCEDURES

Each patient underwent a standardised interview, concerning demography, sexual and venereal disease histories, and present illness, and an examination of the genitals and inguinal lymph nodes.

Urethral exudates (0.05-0.1 ml) were obtained from the urethral meatus by gentle aspiration with a tuberculin syringe (without needle) and transferred to a pyrogen-free plastic test tube containing 1 ml of pyrogen-free water. All specimens were frozen at -20°C before being tested by the limulus assay.

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For culture purposes, a sterile, calcium alginate-tipped, wire, urethrogenital swab (Wilson Diagnostics) or sterile, bacteriological loop was passed 3-4 cm into the urethra. The samples were streaked directly on to Thayer-Martin media and incubated for 48 hours at 35°C in 5% carbon dioxide. No cultures for viruses or chlamydiae were carried out.

The same procedure was repeated to obtain a specimen for Gram-staining and microscopical examination.

LABORATORY METHODS

Gram-stained smears of urethral exudate were examined under $\times 1000$ magnification oil immersion for the presence of polymorphonuclear cells and Gram-negative diplococci. (One microscope was used by one observer.) The results of the smears were considered positive if typical Gram-negative diplococci were seen, whether located intracellularly or extracellularly. A presumptive identification of isolates of *Neisseria gonorrhoeae* was based on characteristic colonial morphology, positive oxidase test, and the presence of Gram-negative diplococci. Isolates were further identified as *N. gonorrhoeae* by typical sugar fermentation reactions. The microscopical diagnosis of NGU required a mean of 10 leucocytes per $\times 1000$ field in a least five fields and the absence of Gram-negative diplococci.

LIMULUS ASSAY

Frozen specimens, of urethral exudate were thawed, vortexed, and diluted in pyrogen-free water to final dilutions of 1/20 and 1/200 of the original sample; 0.2 ml of the diluted specimens was added to single-test Pyrotest™ vials (Difco, by courtesy of A. L. Lane), mixed incubated at 37°C in a water bath for one hour, and read. A firm opaque gel which remained adherent to the bottom of the vial when inverted through 180° was interpreted as a positive test result; the absence of firm gelation was interpreted as a negative result. Known positive and negative control samples were also tested with each assay. The limulus assays were read without previous

knowledge of the microbiological findings. The sensitivity of the limulus test was determined by the detection of less than 0.1 ng/ml *Escherichia coli* endotoxin.

ANALYSIS OF DATA

The results of the limulus assay, Gram-stained smears, and cultures were entered into a Hewlett-Packard Model 9825A programmable calculator for subsequent determination of correlation. Yates's χ^2 method of analysis was used for the determination of statistical significance.

Results

A comparison of the results of the limulus assay for specimens at a 1/200 dilution with those of the Gram-stained smears of patients with and without culture-positive gonococcal urethritis are shown in the Table. Of 73 patients with culture-positive gonorrhoea, 73 (100%) had positive limulus test results and 72 (99%) positive Gram-stained smear results; of 27 patients with probable nongonococcal urethritis, 26 (96%) had negative limulus test results and 27 (100%) negative Gram-stained smear results ($P < 0.001$). Testing of the exudates at a 1/20 dilution gave positive assay results in five (19%) of the 27 patients with nongonococcal urethritis. On retesting at 1/100 and 1/200 dilutions, all the specimens gave negative results to the limulus assay. Only one patient with a negative smear result and a negative culture result for *N. gonorrhoeae* had a positive result to the limulus assay at a 1/200 dilution; on retesting, this specimen produced a positive result at a 1/800 dilution.

Discussion

The outer membranes of *N. gonorrhoeae* are known to contain protein, lipopolysaccharide, and loosely bound lipids (Johnston and Gotschlich, 1974; Wolf-Watz *et al.*, 1975). Recently, Rice and Kasper (1977) have shown that gelation of limulus lysate was caused by the intact outer membranes of *N. gonorrhoeae* and each of its constituents at concentrations as low

Table Results of Gram-stained smears and limulus assay for patients with culture-positive gonococcal and nongonococcal urethritis

Diagnosis	No. of patients	Gram-stained smears		Limulus assay*	
		Positive	Negative	Positive	Negative
Gonococcal urethritis	73	72	1	73	0
Nongonococcal urethritis	27	0	27	1	26

*Original sample diluted 1/200

as 0.7 ng/ml of intact outer membrane, 0.08 ng/ml of protein fraction, and 0.16 ng/ml of lipopolysaccharide fraction. Thus, with the high sensitivity of the limulus assay for components of *N. gonorrhoeae* false-negative results for urethral samples would be unlikely to occur in cases of uncomplicated gonococcal urethritis. Our findings support this hypothesis. In those patients with culture-positive gonococcal urethritis dilutions of 1/200 of the urethral samples produced positive limulus reactions consisting of solid, opaque gelation in every instance.

The potential aetiological agents in NGU have been the focus of recent intense bacteriological, virological, and serological studies (Holmes *et al.*, 1975). The hypothesis that *Chlamydia trachomatis* and *Ureaplasma urealyticum* are the most common urethral pathogens in NGU has been supported by considerable evidence (Wong *et al.*, 1977; Bowie, 1978). *Corynebacterium genitalium* type 1 (Furness *et al.*, 1977), and other bacteria (Bowie *et al.*, 1977), herpesvirus hominis (Jeansson and Molin, 1960), and *Trichomonas vaginalis* (Catterall, 1960) have also been implicated as possible aetiological agents in NGU. Although documented evidence is lacking, it is possible that within the spectrum of agents associated with NGU some could elaborate substances which would produce positive limulus test results. The fact that *C. trachomatis* has a cell wall with antigenic properties similar to but not identical with that of Gram-negative bacteria (Kuo *et al.*, 1977) supports that possibility. Compounds other than bacterial endotoxins (Elin and Wolff, 1973; Wildfeuer *et al.*, 1974) can also give positive results to the limulus test.

Our observations indicated that such potential substances existed in the exudates of some of our patients with NGU. With the exception of one case, however, dilutions of 1/200 of the urethral samples resulted in negative limulus reactions. One specimen showed no bacterial forms on a Gram-stained smear, was culture-negative for *N. gonorrhoeae*, and produced a positive limulus test result at a dilution of 1/800. Further questioning of this patient with a false-positive result to the limulus test elicited that he had received antibiotic therapy for gonorrhoea on eight occasions in three years, had last been treated three months previously and had access to tetracycline. While the reasons for this discrepancy are speculative, the possibilities of sampling-error, unreported antibiotic usage, or contaminated (pyrogen-containing) equipment may provide an explanation.

In the present study, the results of the limulus test on diluted samples of urethral exudate correlated with those of bacteriological methods of diagnosis of urethral gonorrhoea in men with an overall accuracy of 99%. These preliminary studies of otherwise

healthy men with exudative urethritis suggest that the limulus lysate assay is rapid, reliable, and sensitive for the detection of *N. gonorrhoeae* and may assist in identifying cases of NGU. With this test as an adjunct to other diagnostic procedures, it may be possible to give specific treatment at the patient's initial visit even before culture results are available.

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